Potent growth-inhibitory effect of a dual cancer-specific oncolytic adenovirus expressing apoptin on prostate carcinoma

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Abstract. Apoptin is a chicken anemia virus-derived, p53-independent, bcl-2-insensitive apoptotic protein with the ability to specifically induce apoptosis in various human tumor cells, but not in normal cells. To explore the use of apoptin in tumor gene therapy, we assessed a recombinant adenovirus expressing the apoptin protein (Ad-hTERTp-E1a-Apoptin) in order to determine its lethal and growth-inhibitory effects on PC-3 and RM-1 cells *in vitro* and its antitumor effect on solid tumors *in vivo*. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), acridine orange (AO)/ethidium bromide (EB), 4'-6-diamidino-2-phenylindole (DAPI), and Annexin V assays showed that Ad-hTERTp-E1a-Apoptin inhibited the proliferation of PC-3 and RM-1 cells *in vitro* by inducing apoptosis of prostate cancer cells, and that this inhibitory effect was dose and time-dependent. In the animal models, Ad-hTERTp-E1a-Apoptin significantly inhibited tumor growth and extended the lifespan of animals. Experimental results indicate that Ad-hTERTp-E1a-Apoptin has a potential application in tumor gene therapy.

Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer-related deaths in men in the US (1). It is estimated that 217,730 new cases of prostate cancer were diagnosed in 2010 alone (2). The therapeutic options for patients with prostate cancer include surgery, radiotherapy and chemotherapy with cytotoxic agents. Despite a palliative benefit, these approaches do not engender a long-term beneficial effect on the overall survival of patients (3). In this context, there is a pressing need to develop more effective therapeutic approaches for end-stage prostate cancer patients and genetic therapies represent promising approaches for the treatment of this neoplasm (4).

Adenovirus-based vectors are the most widely used cancer gene delivery platforms (5); however, specificity and efficacy are major challenges for this therapeutic strategy (5). Of the existing adenovirus technologies, the utility of conditional replication-competent adenoviruses (CRCAs) provides an optimum approach. In our previous studies, using the RAPAd.I system, we constructed a dual-specific antitumor CRCA, designated Ad-hTERTp-E1a-Apoptin, incorporating the tumor-specific promoter hTERTp and the specific antitumor gene apoptin (6). This CRCA has the ability of both tumor-specific growth inhibition and tumor-specific replication. Further investigation showed that Ad-hTERTp-E1a-Apoptin had a significantly greater antitumor activity than replication-defective adenoviruses (Ad-CMV-Apoptin and Ad-CMV-EGFP) (5).

Apoptosis is frequently impaired in many human tumors, and is also an important mechanism in chemotherapy-induced tumor cell death. Therefore, the modulation of apoptosis by targeting pro-apoptotic and anti-apoptotic proteins may be a powerful and effective method for treating cancer (5). Apoptin, a protein derived from chicken anemia virus (CAV), selectively induces apoptosis in a wide variety of transformed cells, but not in primary cells (6-9).

In this study, we used a recombinant adenovirus expressing the CAV apoptin (Ad-hTERTp-E1a-Apoptin) to infect prostatic carcinoma PC-3 and RM-1 cells, and prostatic carcinoma models with RM-1 cells in C57BL/6 mice. We then tested the lethality and effects of Ad-hTERTp-E1a-Apoptin on PC-3 and RM-1 cells *in vitro* and investigated the antitumor effect of

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Ad-hTERTp-E1a-Apoptin on solid tumors *in vivo*. Our study provided a new strategy for research on gene therapy in prostatic carcinoma.

Materials and methods

Materials. The human prostate cell line PC-3, and the murine cell line RM-1, were obtained from the Cell Bank of Type Culture Collection, Chinese Academy of Sciences, Shanghai, China. Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), and Roswell Park Memorial Institute medium 1640 (RPMI-1640) were bought from Gibco, USA; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), ethidium bromide (EB), and acridine orange (AO), from Sigma, USA; and 4'-6-diamidino-2-phenylindole (DAPI) and Annexin V apoptosis assay, from BioVision, USA; six-week-old C57BL/6 mice were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China. The recombinant adenoviruses Ad-hTERTp-E1a-Apoptin, Ad-hTERTp-E1a-EGFP, Ad-CMV-Apoptin, and Ad-CMV-EGFP were constructed and saved in our laboratory (5) (Fig. 1).

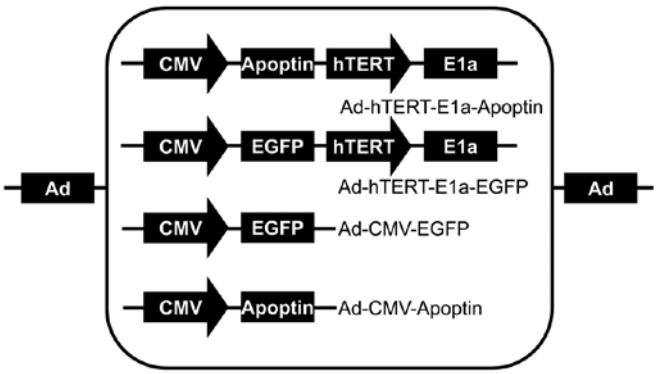

Figure 1. Schematic of recombinant adenoviruses. Schematic diagram depicting the organization of elements in the recombinant adenoviruses. The E1a gene is under the control of the hTERT promoter or the CMV promoter, and apoptin is controlled by the hTERT promoter or CMV promoter. We constructed dual-specific antitumor CRCAs, designated Ad-hTERTp-E1a-Apoptin and Ad-hTERTp-E1a-EGFP, using the RAPAd.I system. The two replication-competent adenoviruses incorporated the hTERTp and E1a gene. The two replication-incompetent adenoviruses (lacking the E1a gene) were Ad-CMV-EGFP and Ad-CMV-Apoptin, in which the CMV promoter drove EGFP and apoptin expression.

Cell culture and viral infection. We incubated $5x10^5$ PC-3 cells in RPMI-1640 and RM-1 cells in DMEM at 37˚C in 5% CO_2; both culture media were supplemented with 10% fetal bovine serum to form complete media. Recombinant adenoviruses were diluted to $5x10^7$ pfu/ml with either DMEM or antibiotic. Infection was performed at a multiplicity of infection (MOI) of 100. The diluted recombinant adenoviruses were inoculated on the cell cultures and maintained at 37˚C in 5% CO_2 for 4 h and then added to their respective complete medium, in which cultivation continued for 48 h.

MTT colorimetric assay. PC-3 and RM-1 cells were seeded in 96-well plates ($5x10^3$ cells/well) and infected with various concentrations (1, 10 and 100 MOI) of recombinant adenoviruses. Viability was measured after 12, 24, 36, 48, 60, 72, 84 and 96 h by treating cells with 20 μl/well MTT (5 mg/ml) and incubating at 37˚C in 5% CO_2 for 4 h. The culture media were removed, and the crystals formed were dissolved by adding 150 μl/well dimethylsulfoxide. Untreated PC-3 and RM-1 cells were used as controls and all measurements were performed in triplicate. The absorbance at 490 nm (A) was measured; untreated cells were used as controls. The percent cell survival was expressed using the following formula: (average absorbance value of control well - average absorbance value of the experimental well)/average absorbance value of control well (5).

AO/EB staining. After a 48-h incubation period, recombinant adenovirus-infected PC-3 and RM-1 cells ($1x10^6$ cells; MOI 100) were trypsinized, washed 2 times in phosphate-buffered saline (PBS), and the cell pellet obtained was resuspended in PBS. To the resuspended solution, we added 2 μl AO/EB solution (AO, 100 μg/ml; EB, 100 μg/ml; dissolved in PBS) and vortexed the resulting sample. Next, 20 μl of the sample was placed on a microscope slide with a cover slip, and images of representative cells were obtained with a digital video camera connected to the 100X objective lens of a fluorescence microscope. The images from the microscope were processed with the Image-Pro Plus (IPP, version 5.0.2) software program.

Annexin V apoptosis assay. After a 48-h incubation period, recombinant adenovirus-infected PC-3 and RM-1 cells ($1x10^6$ cells; MOI, 100) were trypsinized, washed once in PBS, and the cell pellet obtained was resuspended in 200 μl binding buffer. To the resuspended solution, 2 μl fluorescein isothiocyanate (FITC)-labeled Annexin V and 2 μl propidium iodide (PI) were added. The resulting mixtures were incubated in the dark for 5 min at room temperature and examined under a laser scanning confocal microscope.

DAPI staining. After a 48-h incubation period, recombinant adenovirus-infected PC-3 and RM-1 cells ($1x10^6$ cells; 100 MOI) were trypsinized and washed once in PBS as above. The cell pellet obtained was resuspended in 200 μl 25% glutaraldehyde, washed 3 times in PBS, resuspended again in 200 μl 100 ng/ml DAPI and a 20 μl aliquot of the resulting solution was placed on a microscope slide, coverslipped and imaged as described previously.

Animal experiments. RM-1 cells were harvested by trypsinization and resuspended in serum-free DMEM after being washed with PBS. The cell concentration was adjusted to $5x10^7$ cells/ml. Within 1 h of harvesting, 100 μl of cell suspension was injected either subcutaneously into the right flank or into the caudal vein of C57BL/6 mice. When the resulting tumors reached a diameter of 2-5 mm (8 days), the mice were randomly divided into six groups of five mice each. Each mouse in five of these groups received treatment consisting of a single intratumoral and a caudal vein injection; the treatments were repeated two times a week for three weeks and then changed to once a week for three weeks. The five treatments used were 100 μl injections of: i) Ad-hTERT-E1a-

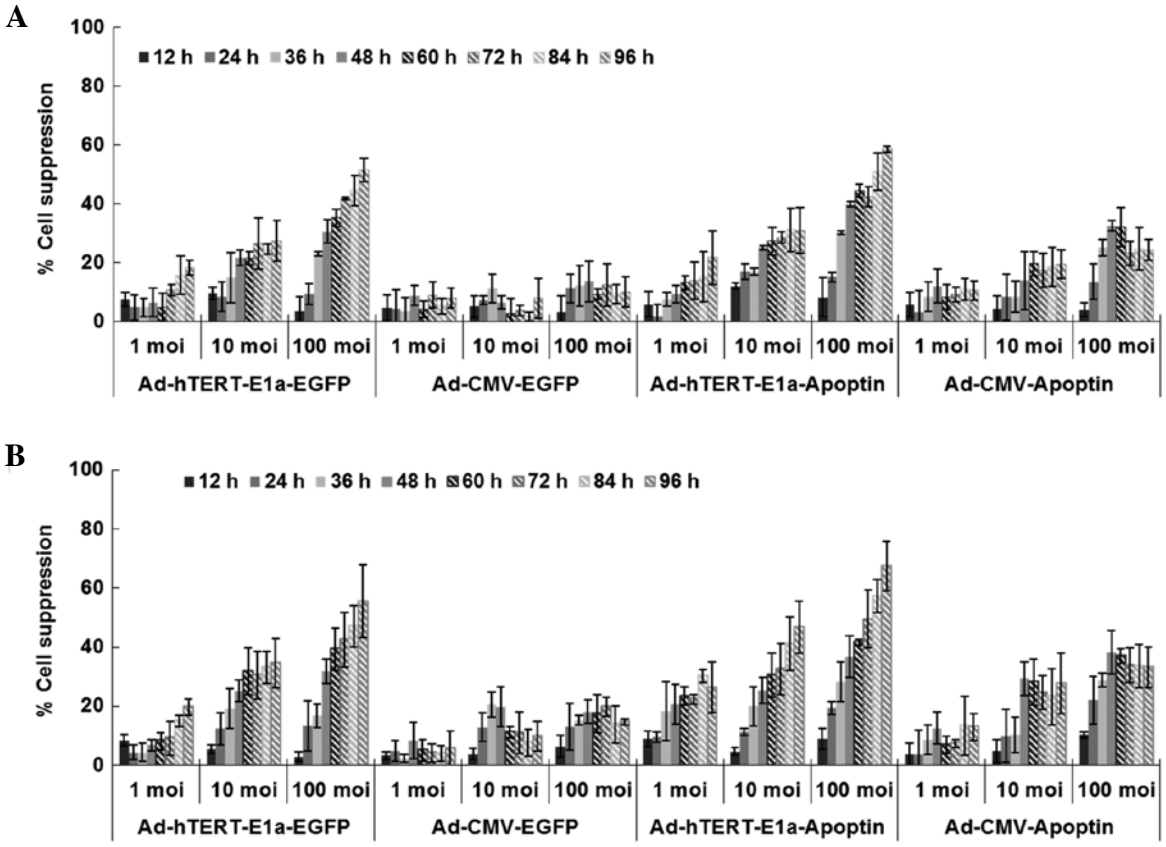

Figure 2. Selective inhibitory effect of recombinant adenoviruses on PC-3 and RM-1 cells. Effects of different MOIs and infection times on: (A) PC-3 and (B) RM-1 cell viability are shown. The cells were seeded in 96-well plates ($1x10^4$ cells/well) 1 day before being infected with various concentrations (1, 10 and 100 MOI) of the indicated adenoviruses. Tumor viability was measured every day over a 4-day period by the MTT colorimetric assay and all measurements were performed in triplicate. Data are presented as mean ± SD. In PC-3 (A) and RM-1 (B) prostate cancer cells, Ad-hTERT-E1a-Apoptin, Ad-hTERT-E1a-EGFP and Ad-CMV-Apoptin infections resulted in significant growth inhibition.

Apoptin alone (10^{11} pfu/mouse in saline), ii) Ad-CMV-Apoptin (10^{11} pfu/mouse in saline), iii) Ad-hTERT-E1a-EGFP (10^{11} pfu/mouse in saline), iv) Ad-CMV-EGFP (10^{11} pfu/mouse in saline) and v) saline. The mice in the sixth group were untreated and served as controls. Tumor size was measured using calipers every 2 days. Tumor volumes were calculated as follows: [0.52 (smallest diameter of tumor)^2 (largest diameter of tumor)] (9). After 63 days, all mice were sacrificed and their cumulative survival was calculated.

Statistical analysis. The statistical significance of differences was determined using one-way analysis of variance (ANOVA), and statistical significance was accepted as $P<0.05$. Log-rank tests were used for survival analysis. Data from all animals are presented in Kaplan-Meier plots.

Results

Lethal effect of Ad-hTERT-E1a-Apoptin on PC-3 and RM-1 cells in vitro. Cell viability was assessed using the MTT colorimetric assay. MTT is taken up into cells by endocytosis or by a protein-facilitated mechanism and reduced, mainly by mitochondrial enzymes, to yield a purple formazan product, which is largely impermeable to cell membranes and therefore accumulates within living cells. Solubilization of the cells liberates the purple product, which can be detected using a colorimetric measurement. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity, which in turn may be interpreted as a measure of cell number/proliferation/viability/survival/toxicity (10).

As shown in Fig. 2, with longer infection times, the growth of PC-3 and RM-1 cells infected with Ad-CMV-Apoptin, Ad-hTERT-E1a-EGFP, Ad-CMV-EGFP and Ad-hTERT-E1a-Apoptin was inhibited. However, cells infected with replication-incompetent adenoviruses (Ad-CMV-Apoptin and Ad-CMV-EGFP) gradually resumed their growth after 48 h. In contrast, Ad-hTERT-E1a-Apoptin and Ad-hTERT-E1a-EGFP were more effective in inhibiting cell growth. Cell viability depended on the MOI of the recombinant adenoviruses to some extent. There was no significant difference in the growth of cells at different infection doses in the first 36 h ($P>0.05$). In contrast, after 48 h, the 100 MOI group showed significantly increased inhibition compared with the 1 MOI and 10 MOI groups. In the 100 MOI group, obvious suppression was seen after 24 h ($P<0.05$). With longer infection times, both Ad-hTERT-E1a-Apoptin and Ad-hTERT-E1a-EGFP were more effective in inhibiting cell growth, but the former was more effective than the latter. In addition, Ad-CMV-Apoptin was more effective than Ad-CMV-EGFP. In PC-3 and RM-1 cells, infection with Ad-CMV-Apoptin at a MOI of 10 or 100 inhibited cell growth by 30-35% after 4 days. Infection with 1 MOI or 10 MOI of Ad-hTERT-E1a-EGFP and Ad-hTERT-

E1a-Apoptin inhibited cell growth by 20-30 and 40-50% after 4 days, respectively and that with 100 MOI almost blocked cell growth (60-70%). Ad-CMV-EGFP, however, did not significantly inhibit cell growth. In conclusion, Ad-hTERT-E1a-Apoptin effectively restricts the growth of cultured PC-3 and RM-1 cells. The interaction between infection time and MOI was complex and synergistic and cell viability showed a non-rigorous dependent relationship with both factors. Therefore, we performed the following *in vitro* experiments 48 h after infection at 100 MOI.

Morphological changes in the recombinant adenovirus-infected PC-3 and RM-1 cells. For the analysis of cell death, we used fluorescent assays of AO/EB double staining. AO is taken up by both viable and non-viable cells, which emit green fluorescence if the dye is intercalated into double-stranded nucleic acid (DNA) and red fluorescence if it is bound to single-stranded nucleic acid (RNA). EB is taken up by only non-viable cells, which emit red fluorescence because of dye intercalation into DNA (11).

Chromatin condensation, nuclear fragmentation and membrane destruction are the hallmarks of apoptotic cells (8). Using AO/EB staining, we analyzed the effects of Ad-hTERTp-E1a-Apoptin and Ad-CMV-Apoptin infections on the nuclear and the membranes of PC-3 and RM-1 cells. As shown in Fig. 3A, normal cell membranes of PC-3 and RM-1 cells were intact and stained bright green with AO. Loss of cytoplasmic membrane integrity resulted in the uptake of EB by Ad-hTERT-E1a-Apoptin- and Ad-CMV-Apoptin-infected PC-3 and RM-1 cells, with orange EB-stained cells dominating over bright green AO-stained cells. Using the AO/EB method, we also quantified the percentage of live, necrotic and apoptotic cells after Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin treatment (Fig. 3B). Infection with Ad-CMV-Apoptin was slightly cytotoxic and the main change in morphology indicated apoptosis more than necrosis (Fig. 3B, middle panels). In contrast, infection with Ad-hTERT-E1a-Apoptin was strongly cytotoxic and apoptosis occurred very quickly, so that the main change in morphology was necrosis rather than apoptosis (Fig. 3B, right panels).

Ability of Ad-hTERT-E1a-Apoptin to induce tumor-specific apoptosis. This assay is based on the ability of the protein Annexin V to bind to phosphatidylserine (PS) exposed on the outer membrane leaflet of apoptotic cells (PS also appears on the necrotic cell surface). In viable cells, PS is located in the inner membrane leaflet, but upon induction of apoptosis, it is translocated to the outer membrane leaflet and becomes available for Annexin V binding. The addition of phosphatidylinositol (PI) enabled viable apoptotic cells to be distinguished from necrotic cells (12).

PC-3 and RM-1 cells infected with Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin were stained with Annexin V-FITC/PI and observed under a laser scanning confocal microscope. Ad-hTERT-E1a-Apoptin- and Ad-CMV-Apoptin-infected cells displayed red fluorescence and fragmented chromatin when stained with PI (Fig. 4A, left panels) and green fluorescence when stained with Annexin V-FITC (Fig. 4A, middle panels). The green fluorescence was mainly concentrated in the cell membrane, a characteristic of phospholipid membranes valgus. PC-3 and RM-1 cells stained with Annexin V-FITC/PI showed a red nucleus (PI) and a halo of green (FITC) on the cell surface, which are indicative of phospholipid membranes valgus and fragmented chromatin. These results indicated that Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin induced apoptosis in PC-3 and RM-1 cells.

The blue fluorescent DAPI nucleic acid stain preferentially stains double-stranded DNA (dsDNA). The stain appears to associate with A/T clusters in the minor groove. Binding of DAPI to dsDNA produces ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. DAPI also binds RNA but through a different mechanism, which is thought to involve A/U-selective intercalation. The DAPI/RNA complex exhibits a longer-wavelength fluorescence emission maximum than the DAPI/dsDNA complex (~500 vs. ~460 nm) and a quantum yield that is only ~20% as high (13).

PC-3 and RM-1 cells infected with Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin were stained with DAPI and observed under a laser scanning confocal microscope. The nuclei of uninfected cells (controls) showed a uniform blue fluorescence and were structurally normal, while those of Ad-hTERT-E1a-Apoptin- and Ad-CMV-Apoptin-infected cells displayed light blue fluorescence and condensed and fragmented chromatin (Fig. 4B). These results indicated that Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin induced apoptosis in PC-3 and RM-1 cells.

Antitumor effect of Ad-hTERT-E1a-Apoptin in vivo. We next examined the antitumor potential of Ad-hTERT-E1a-Apoptin in the RM-1 tumor model. The growth kinetics of the tumors treated with intratumoral injections are shown in Fig. 5A. Compared with the saline control and Ad-CMV-EGFP groups, the recombinant adenovirus groups showed suppression of tumor growth; this effect was seen after the first three injections and continued up to the end of the treatment period. However, soon after the last injection, tumor growth gradually resumed in the recombinant virus groups, but was slowest in the Ad-hTERT-E1a-Apoptin and Ad-hTERT-E1a-EGFP groups. The growth kinetics of the tumors treated with intravenous injections is shown in Fig. 5B. Compared with the tumors in the saline controls and Ad-CMV-EGFP-infected groups, those in the recombinant adenovirus groups were suppressed after the first three injections. Tumor suppression continued up to the end of the treatment period. However, soon after the last injection, the Ad-CMV-Apoptin- and Ad-CMV-EGFP-infected tumors gradually resumed their growth. Most of the Ad-hTERT-E1a-Apoptin- and Ad-hTERT-E1a-EGFP-infected tumors also resumed growth, but these grew more slowly. The tumors in the intravenous injection groups grew more rapidly than those in the intratumoral injection groups. The main cause of this difference may be the faster effect of direct intratumoral injection than of intravenous injection. We also evaluated the ability of the recombinant adenoviruses to prolong the survival of the tumor-bearing mice (Fig. 5C). All saline-, Ad-CMV-Apoptin- and Ad-CMV-EGFP-treated animals died between 26 and 52 days after intratumoral injection. In contrast, 60 and 80% of Ad-hTERT-E1a-EGFP- and Ad-hTERT-E1a-Apoptin-infected animals, respectively, were still alive at that point (Fig. 5C). Mouse survival analysis

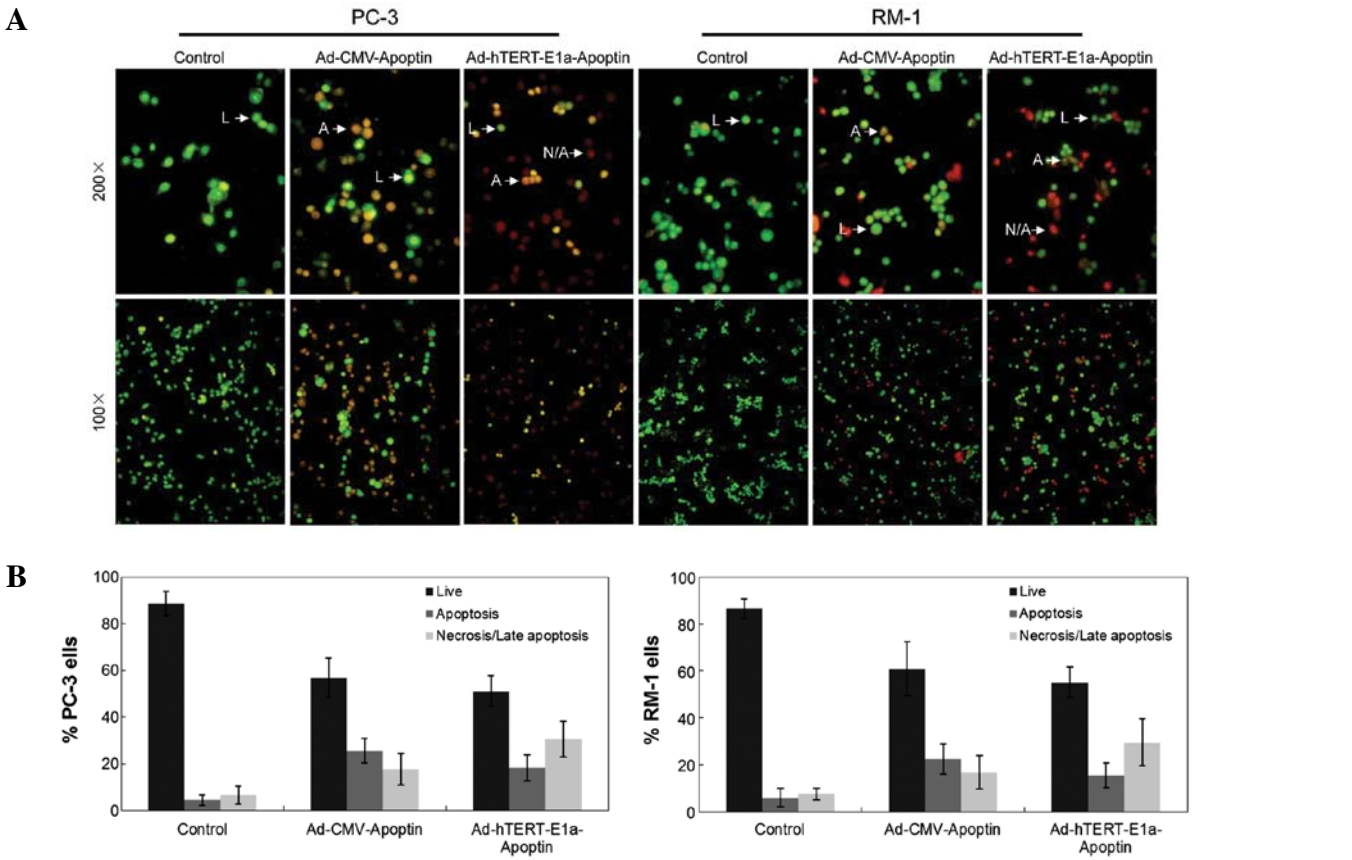

Figure 3. Selective induction of apoptosis in prostate cancer cells by Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin. (A) Cells at $1x10^6$ cells/well were transduced with Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin for 48 h, stained with AO/EB and their morphology was assessed immediately using fluorescence microscopy (x200 and x100). (B) IPP analysis of PC-3 and RM-1 cells infected with Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin. L, normal cell; A, apoptotic cell; N/A, necrotic/apoptotic cell.

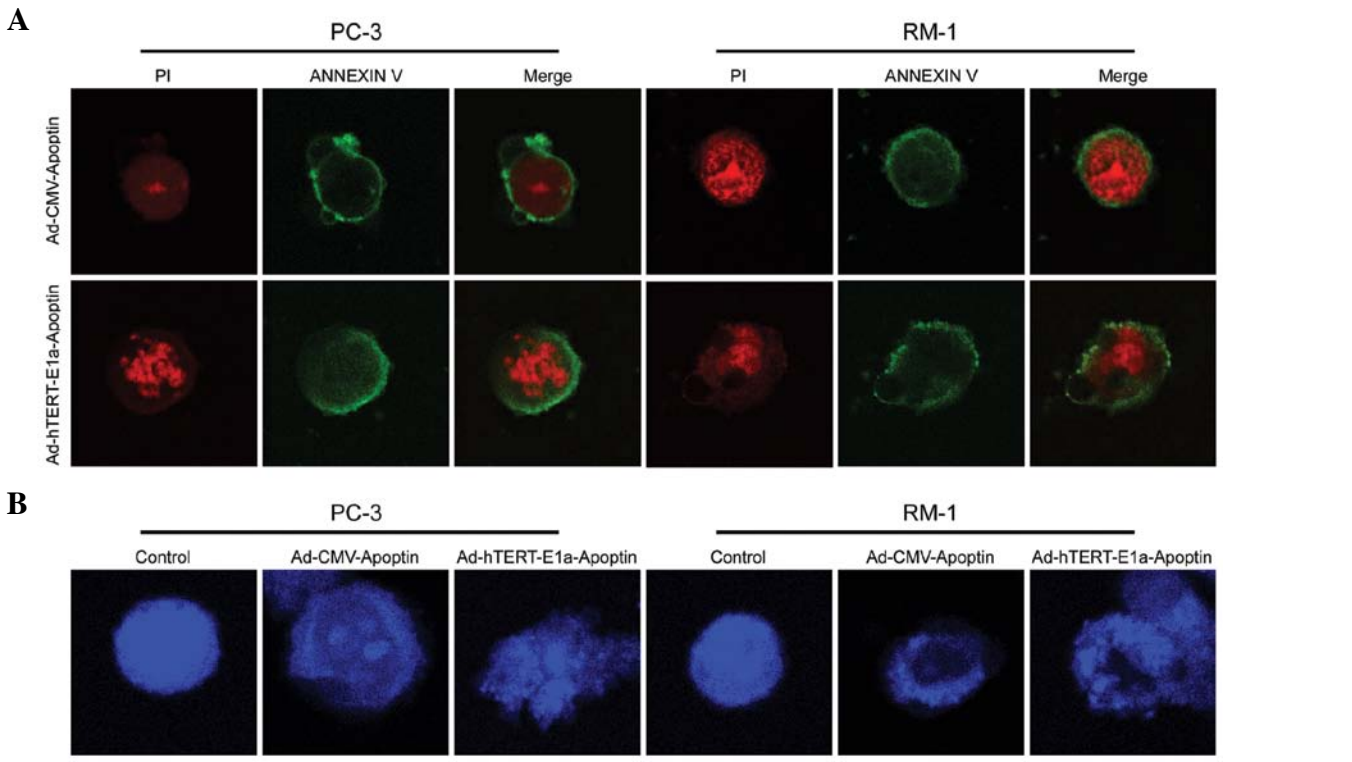

Figure 4. PC-3 and RM-1 cell death induced by Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin. Cells at $1x10^6$ cells/well were transduced with Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin for 48 h, stained with Annexin V (A) or DAPI (B) and their morphology was assessed immediately under a laser scanning confocal microscope.

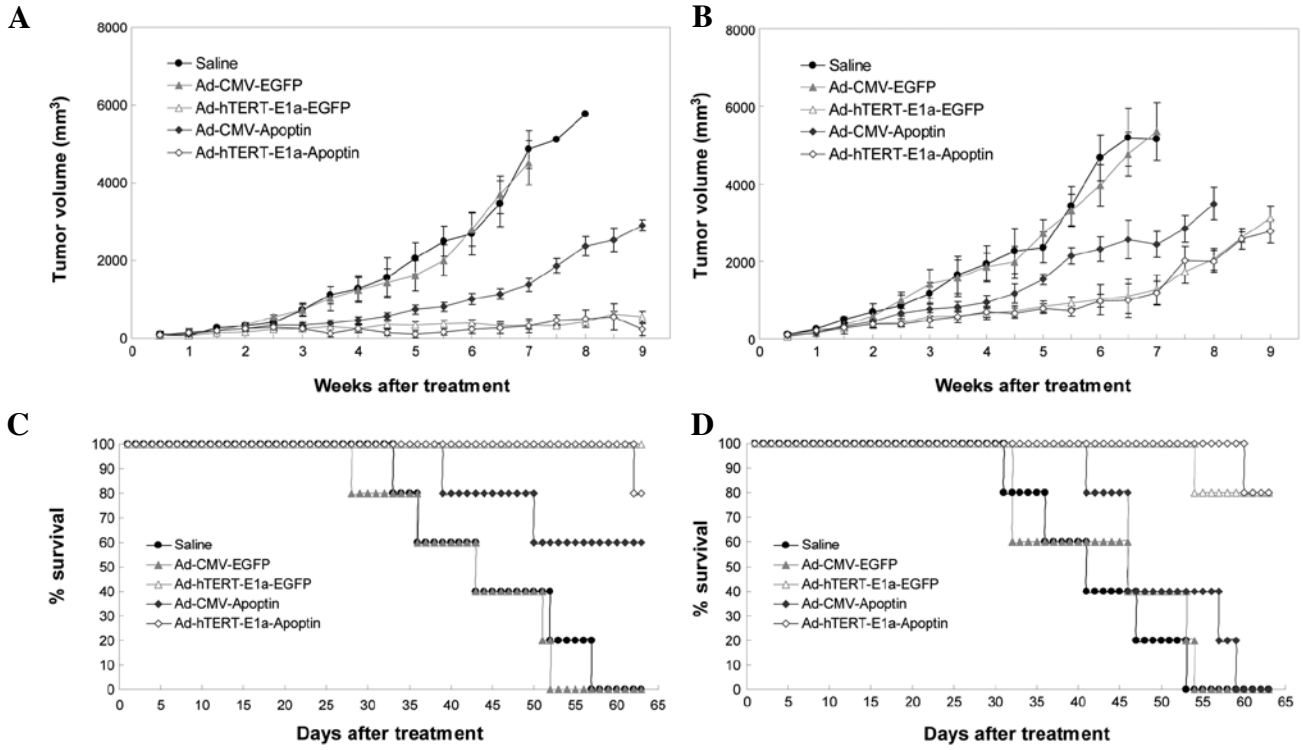

Figure 5. Ad-hTERT-E1A-Apoptin-induced suppression of prostate cancer in the C57BL/6 mouse model. (A) Tumor growth kinetics in mice that received intratumoral injections. (B) Tumor growth kinetics in mice that received intravenous injections. (C) Survival curve of mice treated intratumorally. (D) Survival curve of mice treated intravenously. The day of the first injection was considered as day 0. Data are presented as mean ± SD (A and B). Ad-hTERT-E1a-EGFP and Ad-hTERT-E1a-Apoptin significantly inhibited tumor growth in both the intratumoral (A) and intravenous (B) injection groups. Although Ad-CMV-Apoptin and Ad-CMV-EGFP had some inhibitory effect on the tumors in both injection groups, the antitumor effects of Ad-CMV-Apoptin and Ad-CMV-EGFP in the systemic delivery group were marginal (A and B). Furthermore, increased mean survival was observed in the Ad-hTERT-E1a-EGFP- and Ad-hTERT-E1a-Apoptin-treated mice in comparison with the saline, Ad-CMV-EGFP- and Ad-CMV-Apoptin-treated mice (C and D).

showed that Ad-hTERT-E1a-Apoptin and Ad-hTERT-E1a-EGFP treatments significantly increased mouse survival in the RM-1 tumor model in comparison with the other recombinant adenovirus treatments and saline treatment (Fig. 5D).

When the experiment was terminated on day 63, 80% of Ad-hTERT-E1a-Apoptin- and Ad-hTERT-E1a-EGFP-infected animals were alive and the median survival time did not differ significantly between these two groups. None of the mice in the other groups were alive at the end of the experiment. In conclusion, inoculation with Ad-hTERT-E1a-Apoptin had significant survival benefits and reduced tumor size *in vivo*.

Pathological examination. In the Ad-hTERT-E1a-Apoptin- and Ad-hTERT-E1a-EGFP-treated groups, tumors lost tissue integrity and showed increased intercellular spaces containing remnants of disintegrating cells (Fig. 6A). None of these changes were seen in the Ad-CMV-EGFP-treated, Ad-CMV-Apoptin-treated, and saline control groups. These results indicated that Ad-hTERT-E1a-Apoptin had antitumor effects on solid tumors. This recombinant adenovirus also significantly decreased the tumor burden of the mice. Ad-hTERT-E1a-Apoptin-infected tumors showed minimal metastatic nodules, unlike the other treatment groups and the control group, which showed severe metastasis. Taken together, the systemic delivery of Ad-hTERT-E1a-Apoptin significantly reduced tumor burden and provided survival benefits in the RM-1 tumor model.

Discussion

Like many cancers, prostate cancer is a complex disease, and different types of therapeutic strategies are required to demonstrate a benefit in a particular patient cohort. Most of the ~29,000 men who succumb to prostate cancer each year in the United States die of metastatic disease, and this highlights the need for better systemic therapies (14). In recent years, with the development of molecular biology, immunology and other related subjects, gene therapy has gradually emerged as a novel antitumor treatment that has a huge advantage (14). Viral vectors are used in the field of gene therapy for their simplicity, stability, ease of operation, efficient capacity and safety without integration and have increasingly attracted the attention and favor of researchers.

Apoptin has gained significant attention in recent years, both as a lead for the development of cancer-specific therapeutics and for its potential use as an indicator of cellular transformation processes (15). Apoptin is a 13.6 kDa viral protein encoded by the VP3 gene of chicken anemia virus and is composed of 121 amino acids (15,16). Because of its small size, the apoptin gene can be inserted into various vectors such as parvoviruses, papovaviruses and adenoviruses (17-20). It induces apoptosis independently of death receptor pathways in a broad range of transformed and cancer cells. Apoptin localizes in the nucleus in cancer cells; however, in non-transformed or primary cells, it is localized to the cytoplasm (20-22). The

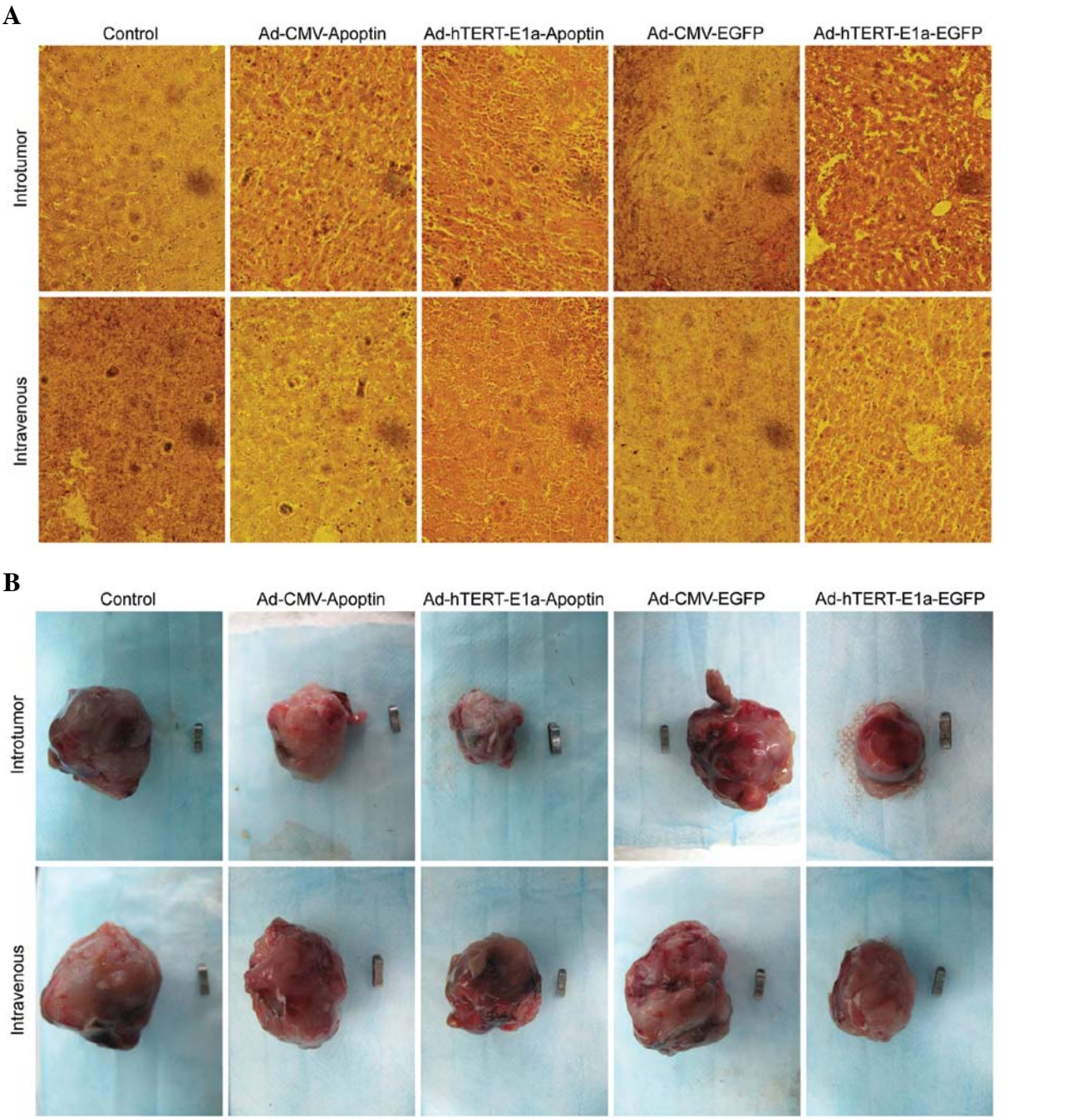

Figure 6. (A) Results of pathological examination of RM-1 tumors in C57BL/6 mice. Tumors treated with the recombinant adenoviruses lost tissue integrity and showed increased intercellular spaces containing remnants of disintegrating cells. (B) Representative images of tumors from the control and treatment groups. With the exception of the Ad-CMV-EGFP-treated group, tumor volume significantly decreased in all recombinant adenovirus groups. Furthermore, intratumorally treated tumors grew more slowly than intravenously treated tumors.

cellular localization of apoptin is influenced by its phosphorylation status at threonine-108. Phosphorylated T-108 inhibits nearby nuclear export signals, leading to nuclear accumulation of apoptin (7,23-25). Apoptin phosphorylation has been proposed to be regulated by Akt-activated cyclin-dependent kinase (CDK)-2 and protein kinase C (PKC) (26-28). Thus, nuclear localization of apoptin and its interaction with specific signaling proteins plays a crucial role in its selective toxicity (6,8,25). Furthermore, apoptin does not induce apoptosis in normal, non-transformed cells such as fibroblasts, keratinocytes, or smooth muscle cells (7).

The hTERT promoter displays high activity in a majority of human cancers but not in most host tissues (29,30) and is considered a good tumor-specific regulator for oncolytic adenoviruses (31). The hTERT promoter can be used to control viral regulatory genes, such as adenoviral E1A, to restrict the replication of oncolytic adenoviruses to malignant cells and tissues. Dual-specificity adenoviral promoters that regulate E1A expression in response to multiple stimuli, e.g., estrogens and hypoxia, have also been described (32). The cancer-specific promoter hTERT can both confer tumor-specific replication and regulate E1A expression and several tumor cell-replicating,

hTERT-driven adenoviruses have been described (31,33). However, none of these viruses combines both promoter elements into a single virus to regulate E1A expression and viral replication. As hTERT is expressed in >90% of cancers (27,28), an oncolytic virus that combines both of these features has the potential to induce oncolytic activity across a broad range of human tumors and tumor cell populations. Cancer gene therapy based on oncolytic adenoviruses has been widely studied in pre-clinical and clinical trials in recent years. In our previous studies, using the RAPAd.I system, we constructed the CRCA Ad-hTERT-E1a-Apoptin incorporating hTERTp and the specific antitumor gene apoptin, which demonstrated tumor-specific growth inhibition (5).

In this study, we described the generation of a recombinant adenovirus vector expressing apoptin and its effects on PC-3 and RM-1 cells *in vitro* and *in vivo* based on its tumor-specific apoptosis-inducing activity. MTT assays indicated that infection with Ad-hTERT-E1a-Apoptin at 100 MOI significantly inhibited the growth of PC-3 and RM-1 cells after 48 h and that the inhibitory effect of Ad-hTERT-E1a-Apoptin was dose- and time-dependent. Infections at 1 or 10 MOI had less effective growth-inhibitory effects. These data indicated that the growth inhibition of PC-3 and RM-1 cells is related to the MOI of Ad-hTERT-E1a-Apoptin and the time period after transduction. In contrast, Ad-CMV-Apoptin- and Ad-CMV-EGFP-infected tumor cells resumed proliferation after 48-h treatment at all MOI doses tested. AO/EB, DAPI, and Annexin V assays indicated that Ad-hTERT-E1a-Apoptin could suppress the growth of PC-3 and RM-1 cells through the induction of apoptosis. Consistent with the MTT assay, the AO/EB, DAPI and Annexin V staining assays demonstrated that Ad-hTERT-E1a-Apoptin and Ad-CMV-Apoptin had the most significant growth-inhibitory effect on PC-3 and RM-1 cells and that Ad-hTERT-E1a-Apoptin was significantly stronger than Ad-CMV-Apoptin.

Analysis of survival and growth tendency of tumors in animal models showed that the tumors in the Ad-hTERT-E1a-Apoptin and Ad-hTERT-E1a-EGFP groups grew more slowly than those in the other groups. All saline-, Ad-CMV-Apoptin- and Ad-CMV-EGFP-treated animals died between 26 and 52 days after the last injection, whereas Ad-hTERT-E1a-Apoptin- and Ad-hTERT-E1a-EGFP-treated mice were still alive at this time point, indicating that Ad-hTERT-E1a-Apoptin could significantly extend the lifespan of animals. Moreover, the tumor size in the Ad-CMV-Apoptin-, Ad-CMV-EGFP-, and saline-treated groups was significantly greater than that in the Ad-hTERT-E1a-Apoptin- and Ad-hTERT-E1a-EGFP-treated groups, indicating that Ad-hTERT-E1a-Apoptin could suppress tumor growth in animal models. In conclusion, Ad-hTERT-E1a-Apoptin was able to inhibit the growth of tumor cells, extend the lifespan of animals and improve survival and quality of life in animal models and has a potential application in tumor gene therapy.

Taken together, gene therapy with apoptin offers unique advantages over current approaches for cancer therapy. The dual-specific recombinant adenovirus Ad-hTERT-E1a-Apoptin induced significant apoptosis of PC-3 and RM-1 cells. The unique action of the Ad-hTERT-E1a-Apoptin may provide a novel and promising candidate for cancer gene therapy in clinical trials for prostate cancer.

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